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Automated analytical systems for drug development studies. I — A system for the determination of drug stability*

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Abstract: An automated system consisting of a pH-stat, microdialysis sampling and a liquid chromatograph was assembled to measure the rate of rapid chemical reactions. 2′,3′,5′-Triacetyl-6-azauridine was used as a model compound to validate the performance of the automated system. Buffer catalysis was minimized by using a non-catalytic concentration of borate buffer along with a pH-stat to maintain the pH during the kinetic run. The microdialysis sampling technique permitted sample quenching and buffering of the solutions to a pH compatible with the LC column materials. The combination of microdialysis sampling and rapid LC analysis allowed reactions with a half-life of approximately 1 min to be sampled every 30 s. The rates of hydrolysis of the drug, measured at different conditions of temperature (37–70°C) and pH (9.0–10.5) using the automated system, compared well with the previously determined values.

Keywords: *Automation; microdialysis; liquid chromatography; kinetics; drug stability; ester hydrolysis; 2′,3′,5-triacetyl-6-azauridine.*

Introduction

Prediction of the stability of drugs and excipients in aqueous solutions has occupied the attention of pharmaceutical scientists since the 1950s. The basic principles developed by the pioneers in the field, Garrett and Higuchi, still form the basis of most stability studies conducted to determine the effects of temperature, pH, ionic strength, buffer type and concentration, and co-solvent type and concentration on the rate of degradation of new chemical entities (NCEs) in aqueous solutions [1–3]. Such studies are necessary to define the stability of NCEs in biological fluids and tissues as well as to identify the conditions most suitable for the development of aqueous formulations.

Assuming that the problems of physical and microbiological instability can be addressed and the degradation products of the drug are not toxic, then the shelf-life of the final product is generally defined as the time for 10% degradation of the active component. A 2-year shelf life is generally regarded as the minimum necessary for the development of a ready to use solution formulation to be commercially viable [1]. Therefore, accelerated stability studies conducted at temperatures greater than ambient and at pH values outside the range acceptable for pharmaceutical formulations ($4 \leq pH_{accept} \leq 9$) form an essential part of the preformulation development of any NCE.

For a non-ionizing drug the effect of pH on the rate of hydrolysis of a drug may be described by a simple phenomenological model in which:

$$k_{obs} = k_H\{H^+\} + k_w + \frac{k_{OH}K_w}{\{H^+\}}, \quad (1)$$

where k_{obs} is the pseudo first-order rate constant, K_w is the ion-product of water, and k_H, k_w and k_{OH} represent the rate constants for the hydrolytic reactions catalyzed by the hydrogen ion, water and the hydroxide ion, respectively. Such a relationship (equation (1)) describes a U-shaped pH profile (Fig. 1) in which the slope

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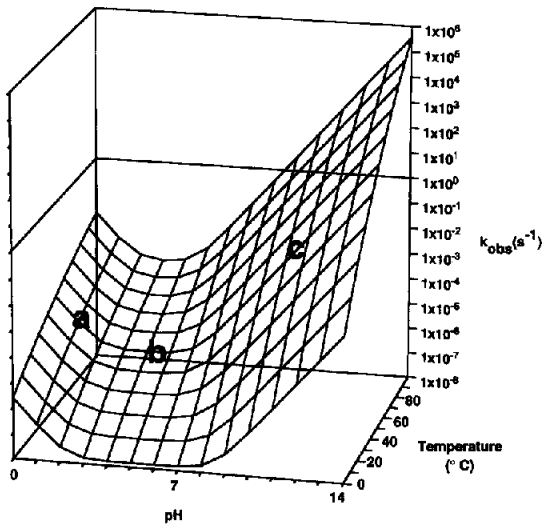


Figure 1
Theoretical three-dimensional relationship between k_{obs} for hydrolysis, pH and temperature. The surface was simulated using: equation (3) and the following values: log A_H = 7.166, log A_w = 6.562, log A_{OH} = 17.78, $E_{a,H}$ = 16 kcal mol^{-1}, $E_{a,w}$ = 18 kcal mol^{-1} and $E_{a,OH}$ = 24. The value of K_w at each temperature was calculated from equation (4) [4] with values as given in the text.

of the relationship between log k_{obs} has a slope of −1 when log $k_{obs} \approx \log k_H\{H^+\}$ (region (a) in Fig. 1), a slope of 0 when log $k_{obs} \approx \log k_w$ (region (b) in Fig. 1) and a slope of +1 when log $k_{obs} \approx \log k_{OH}K_w/\{H^+\}$ (region (c) in Fig. 1). The second extrinsic factor that may be used to accelerate the rate of hydrolysis in a predictable fashion is temperature, which is related to the rate constant, k_i, by the Arrhenius equation:

$$k_i = A_i e - \frac{E_{a,i}}{RT}, \qquad (2)$$

where R is the gas constant (1.987 cal mol^{-1} deg^{-1}, or 4.321 J mol^{-1} deg^{-1}), $E_{a,i}$ is the activation energy, A_i is the frequency factor and T is the absolute temperature. Substituting equation (2) into (1) gives equation (3) which defines the three-dimensional space relating the apparent rate constant for the loss of the drug, to the pH of the solution and the temperature (Fig. 1)

$$k_{obs} = A_H e^{-(E_{a,H}/RT)}\{H^+\} + A_w e^{-(E_{a,w}/RT)} + \frac{A_{OH}K_w e^{-(E_{a,OH}/RT)}}{\{H^+\}}. \qquad (3)$$

Equation (3) contains six unknowns (A_H, $E_{a,H}$, . . . etc.), which implies that the k_{obs}:pH:T surface can be defined with a minimum of six experiments, i.e. by measuring k_{obs} at pH_1:T_1 and pH_1:T_2 (to define region (a), Fig. 1), pH_2:T_3 and pH_2:T_4 (to define region (b), Fig. 1) and pH_3:T_5 and pH_3:T_6 (to define region (c), Fig. 1). (The value of K_w at any temperature may be determined from the equation:

$$\log K_w = A + \frac{B}{T} + \frac{C}{T^2} + \frac{D}{T^3} + \left(E + \frac{F}{T} + \frac{C}{T^2}\right)\log \rho, \qquad (4)$$

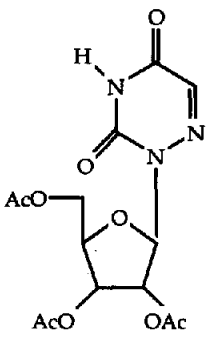
where A = −4.098, B = −3245.2, C = 2.2362 × 10^5, D = −3.984 × 10^7, E = 13.957, F = −1262.3, G = 8.5641 × 10^5 and ρ = density of water [4].) Of course, an important prerequisite of an accelerated stability study conducted at elevated temperature is that the mechanism of degradation does not change with temperature.

Once a stability indicating assay has been developed, liquid chromatography being the method of choice, the characterization of the solution stability of an NCE is typically conducted in two stages. The first stage of the study generally involves experiments conducted at the extremes of pH and elevated temperatures define regions (a) and (c). The data obtained in the first stage may then be used to predict the long-term storage conditions and sampling intervals for the characterization of region (b), which will normally contain the pH of maximum stability.

In principle, defining regions (a) and (c) (Fig. 1) is straightforward because the rate of reaction will be relatively rapid compared with region (b) and the data may be collected quickly. However, the process of defining the stability of a drug under conditions of specific acid (region (a)) or specific base (region (c)) catalysis is complicated by the need to maintain the pH constant with buffers, which may accelerate the reaction by general acid or general base catalysis. Thus the rate of reaction at each temperature and pH has to be determined at several buffer concentrations to obtain the buffer-independent rate constant. Furthermore, several range-finding experiments are often conducted before suitable experimental conditions are identified. Although conceptually simple, preformulation stability studies are experimentally tedious because each step (preparation and sampling

of the solutions, quenching the reaction and analysis of the sample) is conducted as a separate, manual process. Clearly automation of drug stability studies has the potential to accelerate the development of NCEs.

Reversed-phase liquid chromatography is the method of choice for drug stability studies in aqueous solution [5]. It is possible to achieve some degree of automation by conducting the reactions directly in sealed vials placed in an autosampler. However, using a dedicated autosampler assumes prior information on the kinetics of degradation. The use of a dedicated autosampler is also limited by the temperature specifications of the instrument. Furthermore such a system cannot be easily adapted to monitor rapid reactions conducted under extreme conditions because the time to prepare the solution and load them into the vials becomes limiting. Manual sampling requires that the reaction be quenched with a suitable buffer to prevent the further reaction prior to analysis of the samples. Quenching of the reaction may also be necessary to protect the liquid chromatography column, which are generally silica based and very susceptible to hydrolysis by alkaline solutions. Recently, Sauter [6] has described an automated system for continuous monitoring the chemical reactions involved in the synthesis of sensitizing and filter dyes for the photographic research community. In that application dilution of the samples was necessary because the high absorbance of the analytes exceeded the upper limit of LC detector. Sauter's automated technique involved a complex system of valves and syringes for the dilution of the samples and the minimum sampling cycle that they could achieve was 2.5 min. They also pointed out that the minimum sample cycle was often limited by the chromatographic run time, which was dependent on the nature of the sample as well as the configuration of the chromatographic system.



2',3',5'-Triacetyl-6-azauridine (1)

Figure 2
Structure of 2',3',5.040,-triacetyl-6-azauridine (1).

Materials and Methods

Materials

2',3',5'-Triacetyl-6-azauridine (Fig. 2) was a gift from Dr William Drell, U.R. Laboratories, La Jolla, CA. Mono- and dibasic sodium phosphate, sodium chloride, borax, and sodium hydroxide were purchased from Fisher Scientific (Fair Lawn, NJ). Water processed through the Milli-Q water system (Waters, Bedford, MA) was used in all the experiments.

Apparatus

The system was divided into three components (Fig. 3): a pH-stat, a microdialysis sampling system and a liquid chromatograph. The drug solution was placed in a jacketed reaction vessel, thermostated (±0.2°C) using a Fisher Scientific Model 801 circulating water bath. A Metrohm (Schweiz, Switzerland) Model 632 pH meter was used with Model 655 Dosimat and Model 614 Impulsomat in stat-mode to maintain the pH of the reaction mixture. The sampling system consisted of a CMA12, microdialysis probe (4 mm) obtained from BAS (West Lafayette, IN) and a Harvard Apparatus (South Natick, MA) Model 44 infusion pump for continuous perfusion of the probe. The dialysate was passed through a 2 μl injection loop mounted on a Valco Instrument (Houston, TX) Model EF 60 on-line injector. The injector was fitted with an electrical actuator for rapid sampling. The LC system consisted of a Beckman (Fullerton, CA) Model 110A pump, a Kratos Analytical (Ramsey, NJ) Model 757 UV detector (set at 254 nm), and a Shimadzu (Kyoto, Japan) Model C-R3A integrator. Two ODS Hypersil columns (A, 5 μm, 150 mm × 4.6 mm i.d.; B, 3 μm, 30 mm × 4.6 mm i.d.) were purchased from Keystone (Bellefonte, PA). Columns A and B were eluted with methanol–phosphate buffer (50 mM, pH 6.3) (43:57, v/v) and (34:66, v/v), respectively.

Kinetic experiments

The reactions were initiated by adding about

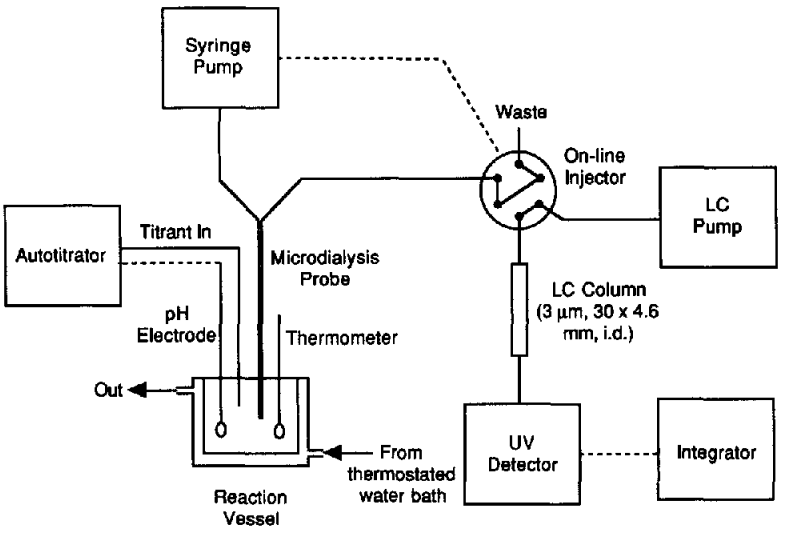


Figure 3
Diagram of the automated system developed for the determination of reaction rates.

5 mg of **1** to 50 ml of the reaction medium, buffered at a desired pH (9.0–10.5) with a non-catalytic amount of borate buffer (approx. 5 mM). A sufficient amount of sodium chloride was added to adjust the ionic strength of the medium to 0.5 and the medium was equilibrated to a desired temperature. The pH of the reaction medium was maintained at a preset value throughout the kinetic run using a pH-stat. A 4 mm CMA12, microdialysis probe was immersed in the reaction medium for sampling. Except when specifically measuring the effects of flow rate, the microdialysis infusion pump was set at 6 µl min^{-1} and the probe was continuously perfused with a 25 mM phosphate buffer (pH 6). The integrator was set up to collect the data for the whole experiment and a print out of peak height responses was obtained at the end of the run. For slower reactions, the data were collected at the end of each injection. The pseudo first-order rate constants (k_{obs}, equation (1)) were measured by fitting the data using least squares linear regression to the following equation

$$\ln \frac{H}{H_o} = -k_{obs}\, t \qquad (5)$$

where H and H_o are the concentrations of the drug at time = t and time = 0, respectively.

Results and Discussion

Model analyte

2′,3′,5′-Triacetyl-6-azauridine (**1**), a prodrug of 6-azauridine, was chosen as a model compound in this study because its hydrolysis has been studied extensively [7] as a function of temperature (37–90°C), pH (1–10), and buffer concentration (0.05–0.15 M). The previous data were obtained by manual sampling of solutions stored at various temperatures in sealed ampules with the pH being maintained by appropriate buffers. The ionic strength in the present investigation and in the previous study was held constant at 0.5 by the addition of sodium chloride.

System characteristics

This paper describes the first phase of the development of an automated reactor interfaced with a liquid chromatograph for the determination of drug stability (Fig. 3). One unique feature of the system is the use of microdialysis sampling, which was found to provide a very convenient method of interfacing the reaction vessel with the liquid chromatograph. Previous analytical applications of microdialysis have involved the use of this technique for the sampling of biological fluids and tissues [8]. The main advantage of microdialysis for the analysis of biological fluids lies in its ease of automation and in its ability to separate rapidly low molecular weight analytes from macromolecules and particulate materials such as cells [8].

In the present system, microdialysis was found to be a very convenient way of rapidly sampling a chemical reaction even though separation from macromolecules was not an

issue. It should be noted that such systems have been used *in vitro* for the determination of protein binding and enzyme kinetics [8]. However, it is believed that this is the first example of the use of microdialysis for the sampling of simple chemical reactions involving solutes of low molecular weight. In the present system the main advantages of microdialysis sampling arises from quenching of the reaction that occurs when the analytes diffuse from the reaction vessel, where the rate of reaction is fast, into the microdialysis perfusate, where the reaction is slow due to the presence of a 25 mM phosphate buffer (pH 6.0). Because the sampling window is very small, the extent of diffusion of phosphate ions into the reaction vessel was small and did not substantially modify the composition of the reaction vessel. This was supported by the observation that no shift in pH was observed when the microdialysis medium was perfused through the microdialysis probe which was placed in the solution containing 5 mM borate buffer. A non-catalytic concentration (5 mM borate) was used together with the autotitrator to maintain the pH value between 9.0 and 10.5. This system allowed the rates of reaction as a function of pH and temperature to be determined without the complicating effects of buffer catalysis. Although no precipitation occurred during the degradation of the model compound, **1** in the present studies, a potential additional advantage of microdialysis to study drug degradation is that it is not necessary to filter the samples prior to analysis.

The maximum sampling interval was limited by the chromatographic run time. Initial studies were conducted with a 15 cm × 4.6 mm (i.d.) column packed with 5 μm ODS Hypersil. With a mobile phase of methanol–phosphate buffer (25 mM, pH 6) (43:57, v/v) and a flow rate of 1.8 ml min^{-1}, the retention time of **1** was 2.0 min, which allowed a run time of 2.5 min. Increasing the concentration of methanol above 43% or the flow rate above 2.0 ml min^{-1} resulted in shorter run times but incomplete resolution of **1** from its degradation products. A minimum run time of 30 s was achieved with a shorter column (3 cm) packed with 3 μm particles of the same stationary phase (ODS) and eluted at 2.5 ml min^{-1} with methanol–phosphate buffer (50 mM, pH 6.3) (34:66, v/v). Under these conditions the retention time of **1** was 25 s.

System validation

Linearity. The linearity of the chromatographic system was determined over the range (1–100 μg ml^{-1}) by manual injection of solutions of **1** dissolved in phosphate buffer (pH 6.0). Each solution was injected in triplicate and the flow rate of the microdialysis perfusate was 6 μl min^{-1}. The mean peak height of **1** was related to the concentration injected by the equation:

$$H = 424.5\,[\mathbf{1}] + 164.7 \quad n = 7 \quad r^2 > 0.999 \tag{6}$$

The RSD value for the peak height at each concentration injected ($n = 3$) was less than 1.5%. The intercept was less than 2% of the peak height response at the highest standard (100 μg ml^{-1}) suggesting a negligible intercept.

Recovery and reproducibility. The recovery of **1** from the microdialysis system was studied as a function of flow rate through the probe and the volume of perfusate introduced into the injection valve. The effect of the volume of perfusate introduced into the 2 μl injection loop was studied from 1 μl, which represented a 50% underfilling of the injection loop, to 5 μl, which represented a 150% overfilling of the injection loop. Table 1 shows that the acceptable precision (RSD ≤ 1.66%) when overfilling the loop was equal to or greater than 50% and that the reproducibility of the microdialysis sampling was independent of the perfusate rate. The very poor reproducibility when the loop was only partially filled was attributed to fluctuations in the stroke of the syringe-pump piston. Clearly, when the loop was overfilled, fluctuations in the flow rate of the perfusion pump were unimportant (Table 1).

Consistent with previous studies, the recovery of the CMA12 microdialysis probe was dependent on the flow rate of the perfusion. Figure 4 shows that the absolute recovery, defined as the concentration of material recovered per unit time, increased with flow rate from 1 to 3 μl min^{-1} and then decreased from 4 to 6 μl min^{-1} due to ultrafiltration of the analyte at higher flow rate. On the other hand, the relative recovery, which is the more important analytical parameter since it influences the sensitivity of the method, decreased with increasing flow rate. Highest sensitivity was achieved if flow rates of 1 μl

Table 1
Reproducibility of microdialysis sampling as a function of the volume introduced into the injection loop at two perfusion rates

Fill volume (μl)	Fill volume/injection volume	RSD* (%) 6 μl min^{-1}	RSD* (%) 2 μl min^{-1}
1	0.5	11.07	9.28
2	1.0	3.23	3.70
3	1.5	1.66	1.37
4	2.0	0.92	1.03
5	2.5	0.47	0.34

*$n = 5$.

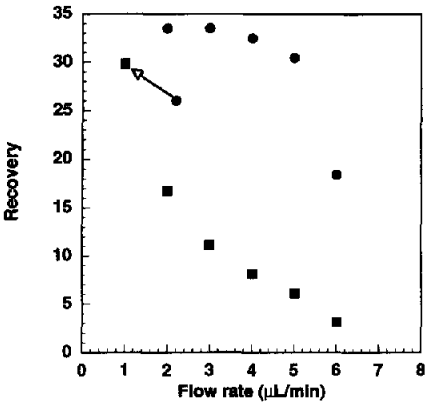


Figure 4
Relationship between the relative (squares) and absolute recoveries (circles) of 1 from the CMA12 microdialysis probe and the flow rate of perfusate. The concentration of 1 was approximately 100 μg ml^{-1} in water. The perfusion medium was phosphate buffer (pH 6.00, 25 mM).

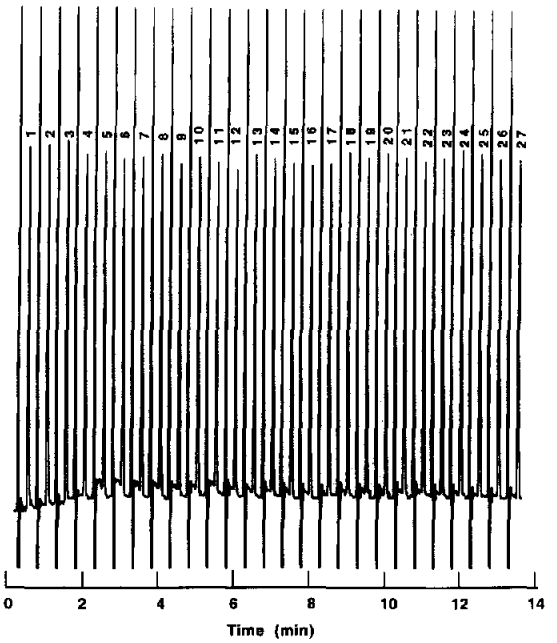


Figure 5
Repetitive analysis of 1 injected every 30 s, showing reproducibility of the system. (RSD = 2.8% ($n = 27$)). Stationary phase: ODS Hypersil column (3 μm, 30 mm × 4.6 mm i.d.). Mobile phase: methanol–phosphate buffer (pH 6.0) (34:66, v/v).

min^{-1} or less were used. However, the flow rate determined the rate of sampling because good precision required at least a 50% overfilling of the injection loop, which had an internal volume of 2 μl. A sampling rate of 2 injections min^{-1} could be achieved with acceptable precision (RSD ≤ 1.66) at a flow rate of 6 μl min^{-1}. Under these conditions, the relative recovery was approximately 3% (Fig. 4). However, Fig. 5 shows the RSD for the peak height of **1** for 27 samples injected every 30 s was 2.8% indicating that the reproducibility of the system was excellent for rapid analyses even though the recovery of **1** was low.

Probe and column stability. The present studies were limited to an evaluation of the kinetics of hydrolysis of a model compound, **1** in alkaline solutions (pH 9.0–10.5) over the temperature range (37–67°C) and no deterioration in probe performance was observed under these conditions. However, the CMA12 probe was found to swell and burst in mildly acidic solutions and could not be used at pH values below 2.0. This is a significant limitation of the present system and studies are being conducted to identify a microdialysis probe that is stable at pH values of less than 2.0.

The maintenance of a buffer capacity in excess of the buffer capacity of the reaction vessel was necessary to avoid the injection of alkaline solutions onto the LC column. When the microdialysis probe was perfused with water rapid column degradation with con-

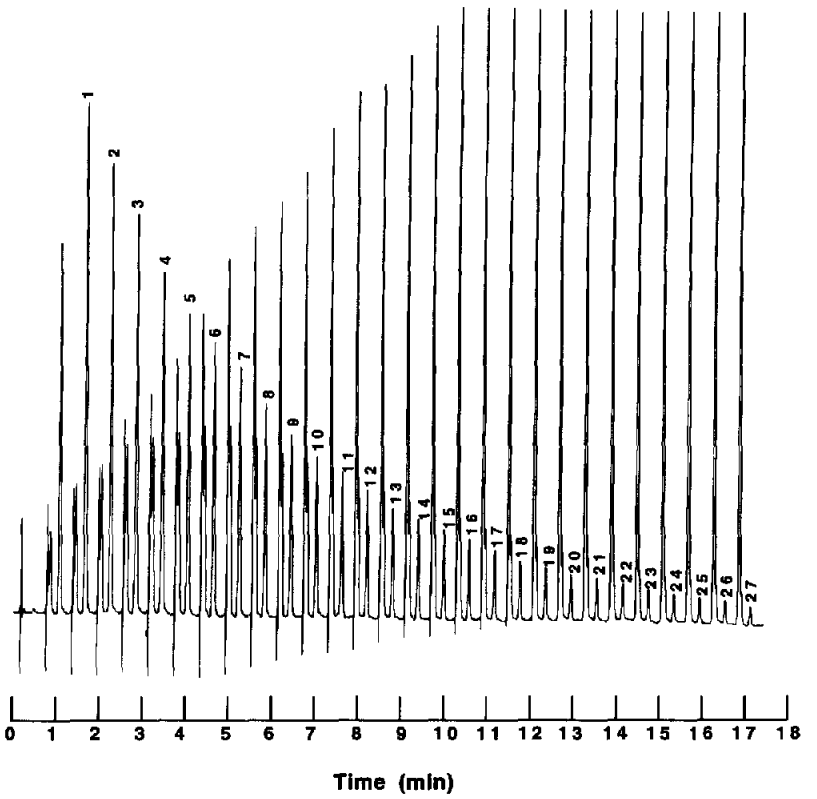


Figure 6
Kinetic run showing the degradation of (**1**) at 37°C and pH 10.5 ($t_{½}$ ~ 3.0 min), conditions as Fig. 5.

comitant deterioration in chromatographic performance was observed when a solution of pH 9.5 was sampled every 60 s. This deterioration in column performance was attributed to hydrolysis of the stationary phase and dissolution of the silica support following repetitive injection of an alkaline solution. No loss in chromatographic performance was observed if the same solution was sampled with a microdialysis perfusate composed of a 25 mM phosphate buffer.

Kinetic studies

Effects of pH and temperature. The applicability of the automated system for the determination of rapid chemical reactions was determined by studying the hydrolysis of **1** under alkaline conditions (pH 9.0–10.5) over the temperature range 37–67°C (Figs 6–8, Table 2). The fastest reaction that could be measured with the present automated system had a half life of 60 s (pH = 9.5, T = 67.0°C) (Table 2). Figures 6 and 7 show that the loss of **1** could be followed to less than 2% of the initial concentration (100 μg ml^{-1}) and that the degradation products of **1** did not interfere

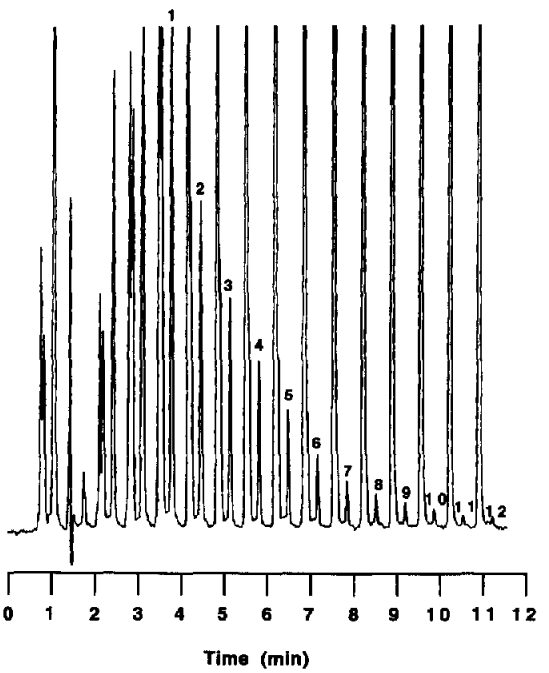


Figure 7
Kinetic run showing the degradation of (**1**) at 67°C and pH 9.5 ($t_{½}$ ~ 1.0 min), conditions as Fig. 5.

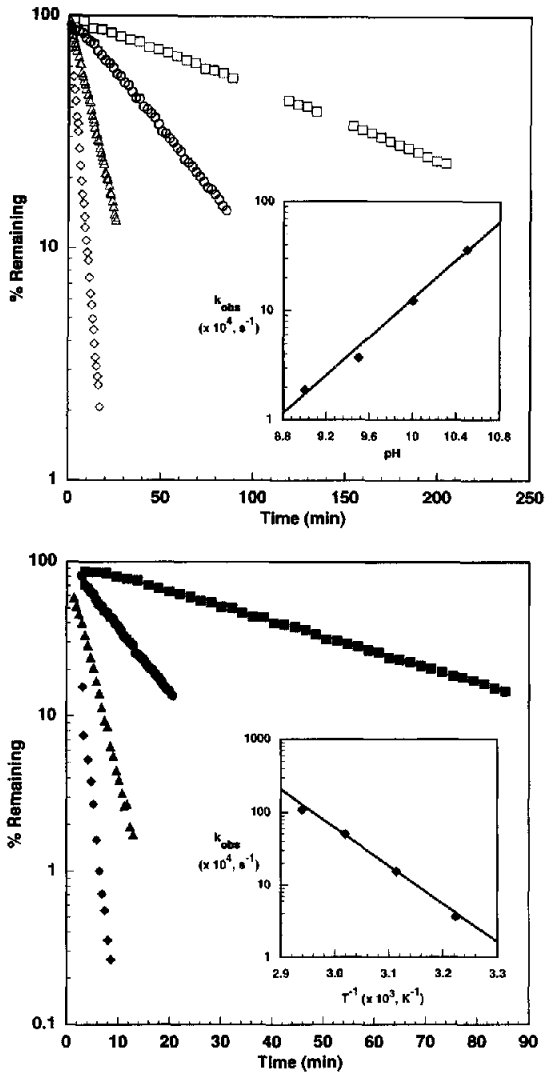


Figure 8
Effect of pH and temperature on hydrolysis of (1). The upper graph shows the effect of pH (squares, 9.0; circles, 9.5; triangles, 10.0; diamonds, 10.5) at a fixed temperature (37.0°C) and the lower graph shows the effect of temperature (squares, 37.0°C; circles, 48.0°C; triangles, 56.0°C; diamonds, 67.0°C) at a fixed pH (9.5). The upper, inset graph shows the relationship between log k_{obs} and the pH of the solution (equation (1)). The lower inset graph shows the relationship between log k_{obs} and the absolute temperature of the solution (equation (2)). The symbols are experimental and the lines are theoretical.

with the analysis. Table 2 shows that the reproducibility of the method for the determination of k_{obs} was excellent. For example the RSDs for the k_{obs} values at pH 9.5 (37.0°C) and pH 10.0 (57.0°C) were 2.37 and 2.06%, respectively.

In all cases the loss of **1** was pseudo first-order (Fig. 8) and the rate of degradation increased with increasing pH and temperature. Figure 8 also shows the linear relationship

Table 2
Pseudo first-order rate constants for the hydrolysis of **1** as a function of pH and temperature (μ = 0.5)

pH	Temperature	k_{obs} ($s^{-1} \times 10^4$)	$t_{1/2}$ (s)
9.0	37.0	1.97	3516
9.5	37.0	3.72	1860
		3.86	1794
		3.89	1782
	48.0	16.34	424.1
	58.0	52.85	131.1
		54.55	127.0
	67.0	116.8	59.6
10.0	37.0	12.87	538.5
		12.39	559.3
		12.30	563.4
		12.65	547.8
10.5	37.0	37.86	183.0

between log k_{obs} and pH (at 37°C), which is consistent with the specific-base catalysis being the predominant mechanism over the pH range 9.0–10.5, i.e.

$$k_{obs} = \frac{k_{OH}K_w}{\{H^+\}} . \qquad (7)$$

Analysis of the data at 37°C (equation (7)) obtained in the present study gave a value of 4.63 M^{-1} s^{-1} for k_{OH}, which compares very favourably with the value of 5.70 M^{-1}, s^{-1} obtained previously by Mummert [7]. Figure 8 shows data relating k_{obs} and temperature plotted according to the Arrhenius relationship:

$$k_{obs} = \frac{A_{OH}K_w e^{-(E_{a,OH})RT)}}{\{H^+\}} . \qquad (8)$$

Analysis of the data at pH 9.5 obtained in the present study gave a value of 24.0 kJ mol^{-1} for $E_{a,OH}$ which compares very favourably with the value of 23.6 kJ mol^{-1} obtained previously by Mummert [7].

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